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Molecular Studies of Knockdown Resistance to Pyrethroids: Cloning of Domain II Sodium Channel Gene Sequences from Insects*

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Abstract: Knockdown resistance (*kdr*) is a target-site resistance mechanism that confers nerve insensitivity to DDT and pyrethroid insecticides. In the housefly, *Musca domestica*, molecular cloning of the *para*-type sodium channel gene has revealed two amino acid mutations that are associated with *kdr* and *super-kdr* resistance phenotypes. Both mutations are located in the domain II region of the channel; Leu1014 to Phe in the hydrophobic segment IIS6 and Met918 to Thr in the IIS4-IIS5 linker. To investigate whether these mutations also occur in other insects, we have designed degenerate primers based on conserved sequences in the domain II region of the sodium channel and used these to PCR amplify this region from insecticide-susceptible strains of eight diverse insect species representing four different insect Orders: *Helicoverpa armigera*, *Plutella xylostella*, *Spodoptera littoralis* (Lepidoptera), *Blattella germanica* (Dictyoptera), *Tribolium castaneum* (Coleoptera), *Myzus persicae*, *Aphis gossypii* and *Phorodon humuli* (Hemiptera). The primers amplified closely related *para*-type sodium channel sequences from each insect with a minimum of 85% amino acid identity between species. All of the sequences contained 'susceptible' Leu and Met residues at the positions associated with *kdr* and *super-kdr* resistance in the housefly. Recent results detailing the presence of a *kdr*-type Leu to Phe mutation in pyrethroid-resistant strains of two important agricultural pests, *P. xylostella* and *M. persicae*, are discussed.

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1 INTRODUCTION

Pyrethroid insecticides combine high insecticidal activity with low mammalian toxicity and environmental persistence and are used widely to control agricultural pests and disease vectors.[1] Their intensive use has led to the development of resistance in many insect species and in several cases this represents a significant threat to their continued effective use.[2] An important mechanism that confers resistance to both pyrethroids and DDT is termed knockdown resistance (*kdr*)[3] and is characterised by a reduced sensitivity of the insect nervous system to these compounds.[4] This type of resistance has been most studied in the housefly, *Musca domestica* L. (Diptera: Muscidae), where several *kdr* variants have been identified, including the more potent *super-kdr* factor that shows greatly enhanced resistance to the more active type II pyrethroids such as deltamethrin.[5]

The primary target site for pyrethroids and DDT is the voltage-sensitive sodium channel of nerve membranes.[6,7] These insecticides alter the gating kinetics of the channel to slow inactivation and thereby prolong the sodium currents associated with membrane depolarisations. This results in uncontrolled bursts of action potentials leading to nerve block and death. Evidence that the resistance of *kdr* insects results from a modification of the sodium channel initially came from cross-resistance studies with certain (site 2) sodium channel neurotoxins[8] and binding studies that indicated a reduced affinity for pyrethroids on the sodium channel of *super-kdr* houseflies.[9] This was further supported by genetic mapping studies that showed close linkage between *kdr* resistance and the *para*-type sodium channel gene, not only in the housefly,[10,11] but also in the tobacco budworm, *Heliothis virescens* F. (Lepidoptera: Noctuidae)[12] and the German cockroach, *Blattella germanica* L. (Dictyoptera: Blattellidae).[13]

To investigate the molecular basis of resistance, we recently cloned the full 6·3 kb coding sequence of the housefly *para*-type sodium channel gene and carried out comparative sequencing studies of susceptible, *kdr* and *super-kdr* strains.[14] We found only two amino acid mutations in the 2108 residue channel protein that correlated with resistance across a range of housefly strains; leucine (position 1014) to phenylalanine in the hydrophobic S6 segment of domain II and methionine (918) to threonine in the nearby domain II S4-S5 linker. The Leu to Phe mutation was found in both *kdr* and *super-kdr* strains whilst the Met to Thr mutation was present only in the *super-kdr* strains.[14] The close correlation of these mutations across a range of housefly strains and their predicted locations within the intracellular mouth of the channel pore (discussed in Reference 14) provide good evidence that they are indeed involved in conferring resistance. We were therefore keen to investigate whether these same mutations are also present in the sodium channel sequences of other insects that have developed *kdr*-like resistance mechanisms. To address this, we describe here the selective polymerase chain reaction (PCR)-based amplification of domain II sodium channel gene sequences containing these two residues from a diverse range of insect species. Although the initial analysis was carried out using insecticide-susceptible strains of each species, the sequence information obtained can be used to rapidly compare the corresponding sequences of pyrethroid-resistant strains/populations of that insect. Recent studies describing the identification of *kdr*-like mutations within this region of the sodium channel, both from our own work and that of others, are discussed.

2 EXPERIMENTAL METHODS

2.1 Insect strains

Pyrethroid-susceptible laboratory strains of the cotton bollworm, *Helicoverpa armigera* (Hübn) (Lepidoptera: Noctuidae), the Eygptian cotton leafworm, *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae), the German cockroach, *Blattella germanica* L. and the red flour beetle, *Tribolium castaneum* Hbst. (Coleoptera: Tenebrionidae) were kindly provided by P. Wege, Zeneca Agrochemicals, Jealotts Hill Research Station, UK. Susceptible strains of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), peach-potato aphid, *Myzus persicae* (Sulz) (Hemiptera: Aphididae) and cotton-melon aphid, *Aphis gossypii* Glov. (Hemiptera: Aphididae) were reared at Rothamsted. Damson-hop aphids, *Phorodon humuli* Schr. (Hemiptera: Aphididae), were collected from the field in the UK during 1995. The response of the *P. humuli* strain to pyrethroids was not investigated, but these insects were collected from wild hops unsprayed with insecticides (H. D. Loxdale, pers. comm.).

2.2 RNA extraction, PCR, cloning and sequencing

Total RNA was extracted from insect material (50–200 mg) ground to a fine powder in liquid nitrogen and homogenised in guanidinium thiocyanate (4 M; 0·5 ml). The homogenate was extracted three times with phenol + chloroform and the RNA precipitated with ice-cold ethanol. The RNA (1–2 μg) was reverse transcribed into single-stranded cDNA using the enzyme Superscript II (Life Sciences) and oligo (dT) primer (200 ng). The primary PCR reaction (50 μl) contained 3 μl cDNA with 0·75 μM primer D1, 1·5 μM primer D3 and 2·5 U Taq DNA polymerase in standard Taq buffer (see Fig. 1 for sequences of the degenerate sodium channel primers D1–D3). The reaction comprised 35 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 3 min with a final extension step of 72°C for 10 min. A

secondary PCR was carried out in 100 μl with 1 μl of the primary reaction as template and 0·75 μM primer D2 instead of D1. The reaction profile was the same except that the annealing and extension steps were reduced to 1 min each. Both the primary and secondary PCRs were 'hot-started' by the addition of Taq polymerase at high temperature (80°C) prior to the cycling.

PCR products were separated on agarose gels (20 g litre^{-1}) and visualised by ethidium bromide staining. Discrete 350 bp amplified fragments from the secondary reactions were excised from the gel and cloned into T-tailed plasmid vector (Invitrogen). Plasmid DNA was sequenced by dye terminator cycle sequencing (PE Applied Biosystems) with the vector M13 forward and reverse primers on an Applied Biosystems 373 automated sequencer. The sequences were processed and analysed using Staden[15] and Wisconsin GCG[16] software packages.

3 RESULTS AND DISCUSSION

3.1 PCR amplification of sodium channel domain II sequences

The strategy for designing oligonucleotide primers to PCR amplify the domain IIS4-IIS6 region of the *para* sodium channel[17] from a range of insect species is shown in Fig. 1. The sequences highlighted (Fig. 1c) were chosen because (i) they flank the two mutations associated with *kdr* and *super-kdr* phenotypes in the housefly (Fig. 1a) and (ii) these sequence motifs are highly conserved in the known channel sequences of both vertebrates and invertebrates (Fig. 1b), thereby increasing the likelihood that they are similarly conserved in other insects. An important exception to this is the *DSC1* sequence, a second putative sodium channel from *Drosophila melanogaster* Meig.,[18] which does have amino acid differences in both of these motifs (Fig. 1b). These differences were deliberately not built into the degeneracy of the primers in order to make them more selective towards the *para*-type sodium channel homologue compared to *DSC1*. The high degeneracy of the 3′ primer D3 (mixture of 2048 sequences) reflects the codon degeneracy of the sequence motif in IIS6 and was unavoidable, as a similarly conserved region of channel sequence was not available downstream within the domain II-III linker.

The presence of introns within this region of the *para* gene from *D. melanogaster*[17] and housefly (unpublished results) meant that we were unable to use genomic DNA as the template for PCR; one of these introns occurs within the D3 primer sequence which would prevent primer annealing. Instead, total RNA was extracted from each of the eight insect species and first strand cDNA used as the template for PCR (RT-PCR). Two rounds of PCR were carried out, with the second reaction using a third 'nested' primer to improve the selectivity of the amplification (Fig. 1c). The first PCR using primers D1 and D3 generally gave a mixture of amplification products within the size range 200–500 bp (not shown). However, the second round of PCR using primers D2 and D3 yielded fragments of the expected size (350 bp) from each of the eight species analysed. These fragments were cloned and sequenced as described in Section 2.2.

3.2 Analysis of domain II sequences

The deduced amino acid sequences of the fragments amplified from each species are shown aligned in Fig. 2, together with the corresponding *para*-type sequences from *D. melanogaster* and housefly. The sequences are clearly very similar with the lowest level of amino acid homology between species being over 80%. This indicates that the primers have indeed selectively amplified the *para*-type sodium channel gene from each species (see above), since any *DSC1* sequences would be expected to be more highly divergent (there is only 60% identity between *para* and *DSC1* within this region).

In general, the sequence similarities reflected taxonomic relationships with the greatest levels of identity observed between species of the same insect Order. For example, the three aphid species (Order Homoptera) *M. persicae*, *A. gossypii* and *P. humuli* are 99% identical, with only one amino acid difference within this region. Similarly, the three Lepidoptera, *P. xylostella*, *H. armigera* and *S. littoralis* also share 99% identity, and the previously published sequences of *D. melanogaster* and *M. domestica* (Diptera) are 97% identical. The sequences of *T. castanenum* (Coleoptera) and *B. germanica* (Dictyoptera) share 92% identity, whilst other inter-species homologies range between 85% (Homoptera against Lepidoptera) and 95% (Diptera against Lepidoptera). Corresponding nucleotide identities are lower, ranging between 72% and 96%.

The eight new sequences described here all contain leucine and methionine residues at the two key resistance positions (highlighted in Fig. 2b), rather than the phenylalanine and threonine mutations found at these positions in *kdr*/*super-kdr* houseflies. This is consistent with the fact that pyrethroid-susceptible strains of each insect were used for this analysis. Moreover, the conservation of these two residues in the *para*-type channel sequences of the diverse range of insects analysed here reinforces the view that the mutations observed at these positions in the *kdr*/*super-kdr* housefly strains are likely to be functionally important for resistance rather than simple polymorphisms between strains.

3.3 *Kdr*-type sodium channel mutations in other insects

The characterisation of domain II sequences from each insect species as described above enables the design of

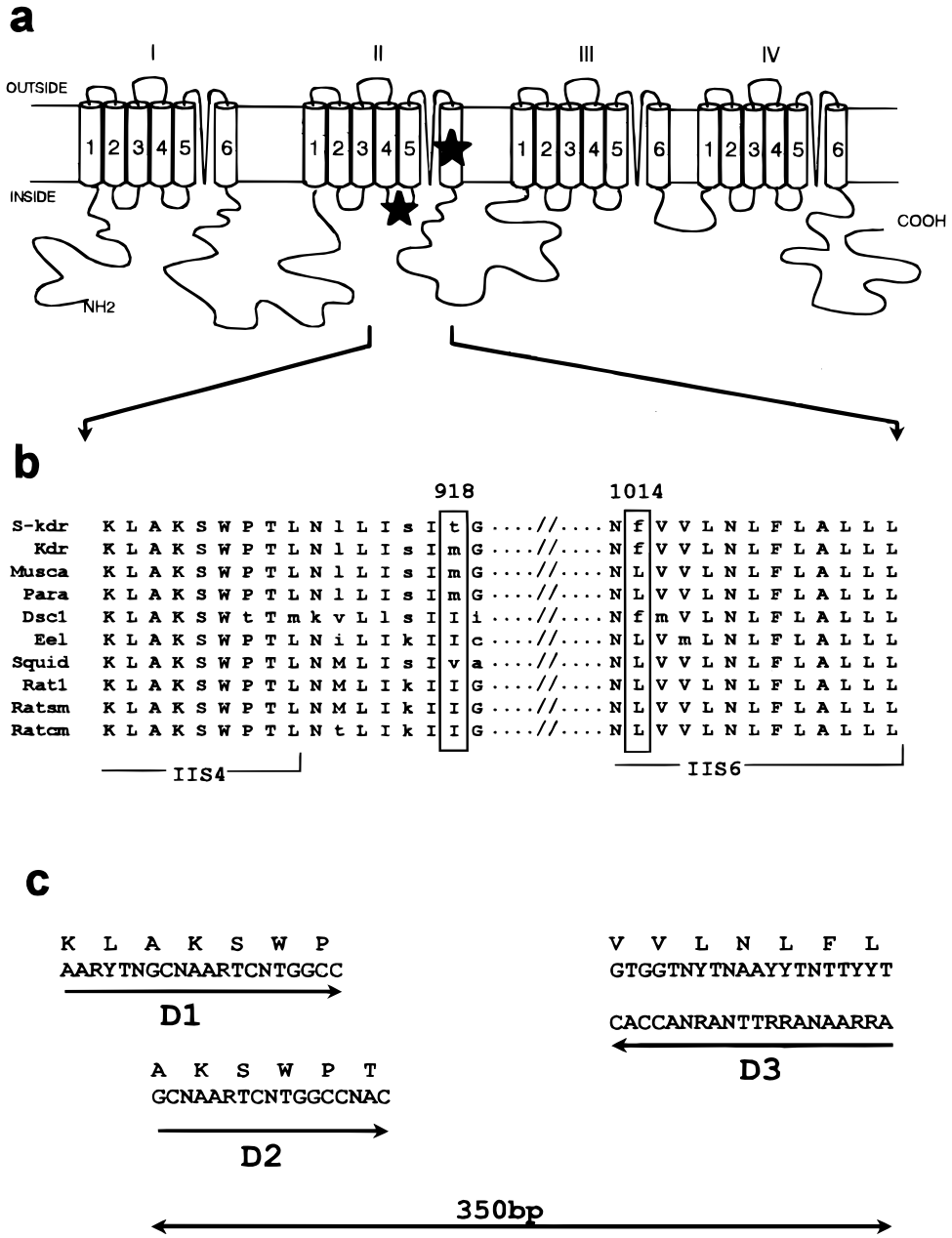

Fig. 1. a: Diagram of the sodium channel showing the four homologous domains (I–IV) and proposed membrane folding of transmembrane regions (S1–S6) within each domain. The positions of the mutations associated with *kdr* and *super-kdr* in the housefly are marked (★). b: Identification of conserved channel sequences that flank the mutations. The aligned IIS4 and IIS6 sequences are from *super-kdr*, *kdr* and susceptible (*Musca*) housefly strains, the *para* and *DSC1* sodium channels of *Drosophila melanogaster*, eel (*Electrophorus electricus*), squid (*Loligo bleekeri*) and rat brain (Rat1), skeletal muscle (Ratsm) and cardiac muscle (Ratcm). The housefly mutations are boxed. c: Sequences of the degenerate primers designed against the conserved sequences and used to PCR amplify this region of the sodium channel gene from different insects. R = A + G, Y = C + T, N = A + C + G + T.

more selective primers for the rapid amplification and sequencing of this region from pyrethroid-resistant strains. In this way, the domain II mutations previously associated with *kdr* resistance in the housefly can be targeted quickly for their presence or absence in other insects. We have recently carried out such an analysis for two of the important agricultural pest species mentioned above, *P. xylostella*[19] and *M. persicae*.[20] In both cases, pyrethroid-resistant strains were found to carry the same leucine to phenylalanine mutation as that described for the *kdr* housefly strains.

For *P. xylostella*, the strain analysed (FEN) was known to have high-level, non-synergisable resistance to pyrethroids and DDT, and was also shown to be nerve-insensitive to deltamethrin by a neurophysiological assay of larvae neuromuscular activity.[19] Hence, the identification of the sodium channel mutation provided confirmation of a *kdr*-type resistance mechanism at the molecular level that had already been implicated from bioassay and physiological data.

In contrast, the identification of this mutation in *M. persicae* was far less expected. Two clones were initially

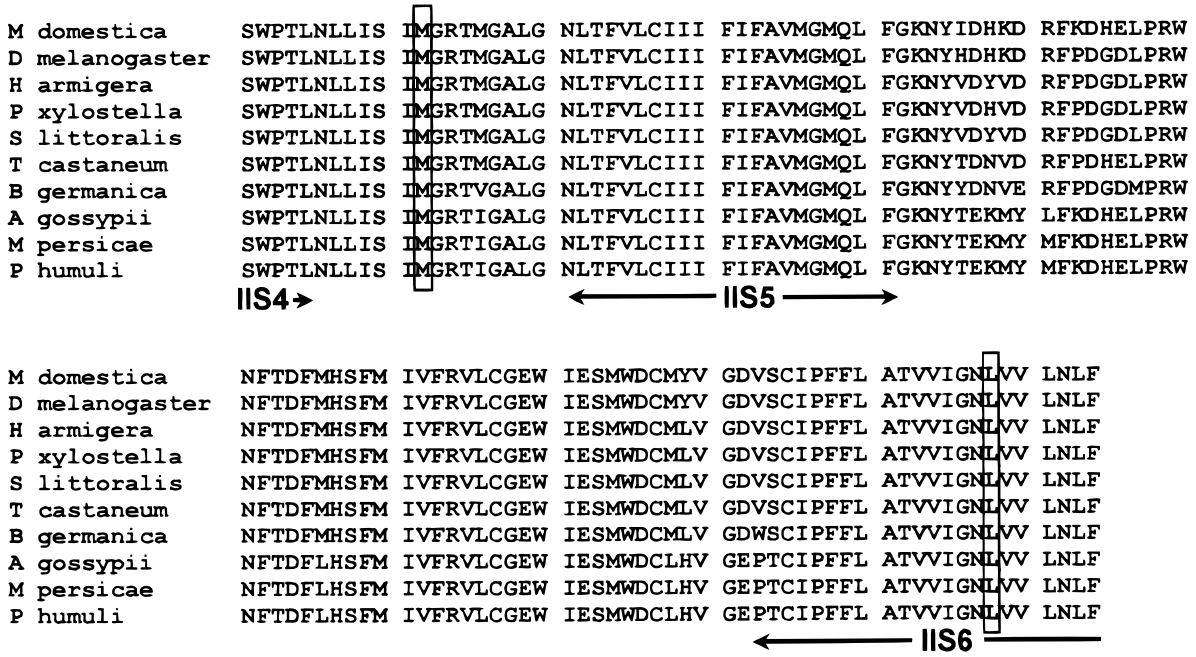

Fig. 2. Amino acid sequences deduced from the 350 bp PCR fragments of each insect species aligned with the previously published *para* sodium channel sequences of *Musca domestica* and *Drosophila melanogaster*. Sequences are from *Helicoverpa armigera*, *Plutella xylostella*, *Spodoptera littoralis*, *Tribolium castanenum*, *Blattella germanica*, *Aphis gossypii*, *Myzus persicae* and *Phorodon humuli*. Positions of amino acid mutations in *kdr*/*super-kdr* houseflies are boxed.

sequenced, 794J and 800F, containing amplified E4 and FE4 esterase genes,[21] respectively. Both clones were known to be resistant to pyrethroids, but this was thought to result solely from the over-production of the E4 and FE4 esterases, conferring broad-spectrum resistance to organophosphates, carbamates and pyrethroids.[22] However, sequence analysis of the domain II region of the sodium channel revealed that the E4 clone, 794J, does in fact contain the *kdr*-associated Leu to Phe mutation, whereas the FE4 clone, 800F, lacks this change.[20] A subsequent re-evaluation of the resistance status of these clones using deltamethrin and DDT supported this finding in that 794J (with the mutation) has greatly increased resistance to both compounds.[20] This suggests that the pyrethroid resistance of 794J stems not just from the esterase-based detoxication, but also from an underlying *kdr*-type nerve insensitivity mechanism involving the sodium channel modification. Furthermore, a more extensive survey of resistance in *M. persicae* has shown that this *kdr*-type mechanism is in fact widely distributed in populations from many parts of the world where it is usually, but not always, linked to the amplified E4 gene locus.[23] The identification and distribution of *kdr* in populations of *M. persicae* clearly has important implications for managing resistance in this pest, and also demonstrates how a molecular approach, such as that adopted here, can generate novel information about the underlying resistance mechanisms that will impact directly on management in the field.

During the course of this work, three reports of *kdr*-like mutations within the sodium channel sequences of other insects have been published. Two of these[24,25] describe exactly the same domain IIS6 Leu to Phe mutation in *kdr* strains of *B. germanica* as that identified in the housefly. The third also reports mutation of this same leucine residue in pyrethroid-resistant populations of *H. virescens*, although in this case the mutation was from Leu to His.[26] Hence the identification of the Leu to Phe mutation in *P. xylostella* and *M. persicae* extends the range of this mutation to five species representing four different insect Orders. The consistent identification of mutations at this one position provides overwhelming evidence that these mutations do indeed constitute the primary cause of resistance, and that the native leucine residue at this position therefore defines part of a critical site for the interaction of pyrethroids with the channel protein. At this stage, we would speculate that the leucine mutation (to Phe or His) represents the primary mutation conferring a base level of resistance to pyrethroids and DDT. However, it seems likely that this can be further enhanced by secondary mutations within this region that further destabilise the interaction of certain pyrethroids. An example of this would be the Met to Thr mutation within the domain IIS4-IIS5 linker, which is consistently found together with the Leu/Phe in *super-kdr* housefly strains[14] conferring enhanced resistance to type II pyrethroids. Given the limited number of insect species and strains that have been analysed so far, it seems unlikely that we have identified the full range of primary and secondary mutations that are capable of conferring this type of resistance. However, given the recent pace of progress is this area and the ability to rapidly sequence selected regions

of the sodium channel gene as described here, it seems unlikely that it will be long before these and perhaps other novel mutations are identified in a much wider range of insect and mite species.

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